HIV-1 Glycan Density Drives the Persistence of the Mannose Patch within an Infected Individual

Karen P. Coss, a Snezana Vasiljevic, b Laura K. Pritchard, b Stefanie A. Krumm, a Molly Glaze, b Sharon Madzorera, c,d,e Penny L. Moore, c,d,e Max Crispin, b Katie J. Doores a

Department of Infectious Diseases, Faculty of Life Sciences and Medicine, King’s College London, London, United Kingdom a; Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford, United Kingdom c; Department of Virology, University of the Witwatersrand, Johannesburg, South Africa d; National Institute for Communicable Diseases (NICD) of the National Health Laboratory Service (NHLS), Johannesburg, South Africa e; Centre for the AIDS Programme of Research in South Africa (CAPRISA), University of KwaZulu Natal, Durban, South Africa c,d,e

ABSTRACT
The HIV envelope glycoprotein (Env) is extensively modified with host-derived N-linked glycans. The high density of glycosylation on the viral spike limits enzymatic processing, resulting in numerous underprocessed oligomannose-type glycans. This extensive glycosylation not only shields conserved regions of the protein from the immune system but also acts as a target for anti-HIV broadly neutralizing antibodies (bnAbs). In response to the host immune system, the HIV glycan shield is constantly evolving through mutations affecting both the positions and numbers of potential N-linked glycosylation sites (PNGSs). Here, using longitudinal Env sequences from a clade C-infected individual (CAP256), we measured the impact of the shifting glycan shield during HIV infection on the abundance of oligomannose-type glycans. By analyzing the intrinsic mannose patch from a panel of recombinant CAP256 gp120s displaying high protein sequence variability and changes in PNGS number and positioning, we show that the intrinsic mannose patch persists throughout the course of HIV infection and correlates with the number of PNGSs. This effect of the glycan density on the processing state was also supported by the analysis of a cross-clade panel of recombinant gp120 glycoproteins. Together, these observations underscore the importance of glycan clustering for the generation of carbohydrate epitopes for anti-HIV bnAbs. The persistence of the intrinsic mannose patch over the course of HIV infection further highlights this epitope as an important target for HIV vaccine strategies.

IMPORTANCE
Development of an HIV vaccine is critical for control of the HIV pandemic, and elicitation of broadly neutralizing antibodies (bnAbs) is likely to be a key component of a successful vaccine response. The HIV envelope glycoprotein (Env) is covered in an array of host-derived N-linked glycans often referred to as the glycan shield. This glycan shield is a target for many of the recently isolated anti-HIV bnAbs and is therefore under constant pressure from the host immune system, leading to changes in both glycan site frequency and location. This study aimed to determine whether these genetic changes impacted the eventual processing of glycans on the HIV Env and the susceptibility of the virus to neutralization. We show that despite this variation in glycan site positioning and frequency over the course of HIV infection, the mannose patch is a conserved feature throughout, making it a stable target for HIV vaccine design.

The HIV envelope glycoprotein (Env) is coated in a dense array of host-derived N-linked glycans. These glycans not only shield conserved regions of the protein from neutralizing antibodies (nAbs), but also act as targets for many of the most broad and potent HIV neutralizing antibodies (1–6). Although HIV Env is glycosylated by the host cell glycosylation machinery, Env glycosylation has been shown to diverge from that typically observed in mammalian cells (1, 7–15). The dense clustering of potential N-linked glycosylation sites (PNGSs) sterically restricts the access of glycan-processing enzymes in the endoplasmic reticulum (ER), which results in a population of underprocessed oligomannose-type glycans (7–17) that is a distinctive feature of HIV Env (2) and is independent of producer cells (18). Site-specific analysis of the glycans on recombinant gp120 shows that these oligomannose-type glycans cluster together on the outer domain (OD) of gp120 (11, 13, 14, 19, 20), and this cluster is often referred to as the mannose patch and is conserved across Env expression systems (including virion-associated Env, SOSIP trimers, and recombinant gp120 monomers) and different geographical clades (7, 15, 17, 18, 20, 21). During expression of both monomeric and trim-
Three main glycan-dependent sites of vulnerability on Env have been identified so far. They include the N332 glycan/V3 loop, which comprises the intrinsic mannosse patch (recognized by, e.g., PGT128, PGT121, 10-1074, and PGT135 [5, 23–25]), but also the N160 glycan/V1/V2 loops (recognized by, e.g., PG9, PG16, PGT145, CAP256-VR2C6.25, and CH04 [5, 26–28]) and the glycans near the gp120/gp41 interface (recognized by, e.g., PGT151, 35O22, 8ANC195 [29–31]). Protein epitopes, such as the CD4 binding site and the membrane-proximal external region (MPER), also show some dependence on N-linked glycosylation. For example, the glycans situated on the rim of the CD4 binding site can modulate the neutralization breadth and potency of CD4 binding site broadly neutralizing antibodies (bnAbs) (22, 32), and the perturbation of gp41 glycosylation has been shown to influence the maximum neutralization of MPER bnAb 10E8 (33).

During infection, HIV Env is under constant pressure from the host immune system, in particular neutralizing antibodies, and as such, the location and frequency of PNGSs often change (3, 34). This observation has led to the concept of the shifting or evolving glycan shield (3). Recent studies aimed at mapping the development of HIV bnAbs in HIV-infected patients has revealed the importance of shifting PNGSs in bnAb development and has shown that immune escape from strain-specific antibodies can lead to formation of bnAb epitopes (35–37). For example, Moore et al. showed in an HIV-infected individual that immune pressure against the N334 glycan in a founder virus led to a shift to the conserved N332 glycan position and subsequent development of an N332-dependent bnAb response in that donor (35). Further, removal of the N276 glycan has been shown to confer sensitivity to germ line variants of CD4 binding site bnAbs, e.g., VRC01 and NIH45-46, indicating that addition of this glycan as a potential escape mechanism is critical for development of a broadly neutralizing CD4 binding site antibody response (38).

Studies comparing Env sequences from donor-recipient pairs and large numbers of acute and chronic viruses have shown that clade C transmitted viruses, and to a lesser extent clades A and D, tend to have shorter variable loops and a lower number of PNGSs than chronic viruses (39–43). These trends are observed for both sexual transmission and mother-to-child transmission; however, the significance of these differences for HIV transmission is not fully understood. Analysis of longitudinal Env sequences over years of HIV infection has shown that there is an increase in both variable-loop length and PNGS frequency, which is reversed in the later stages of infection (44, 45). It is proposed that this initial increased glycosylation shields neutralizing protein epitopes from the host immune system, which also wanes during late infection (3, 34, 46, 47). Although these studies defined changes in the position and frequency of PNGSs over the course of HIV infection, the effects of these changes on the composition of the glycans present on Env, in particular the persistence of the intrinsic mannose patch, have not yet been determined.

Here, we use longitudinal Env sequences from a clade C-infected donor, CAP256, to determine the change in glycan shield composition and the abundance of oligomannose-type glycans in the intrinsic mannose patch over the course of HIV infection and to relate these changes to variable-loop length, frequency of PNGSs, and neutralization sensitivity by a panel of HIV bnAbs. The development of the bnAb response in donor CAP256 has been extensively studied and is mediated by bnAbs directed to the V1/V2 region on Env (27, 48, 49). This patient was infected with a clade C virus and later became superinfected with a second, unrelated clade C virus between weeks 13 and 15, leading to Env recombination (27, 48, 49). The viral population early in infection was predominantly made up of the superinfecting (SU) virus with only the V1/V2 and gp41 C terminus mostly derived from the primary infecting (PI) virus (49), but later in infection, multiple different recombinant forms existed. Escape from the bnAb response occurred through mutation in V2, in particular at residues R166 and K169 (27, 48, 49).

Here, we show that although the number of PNGSs varies by up to five, the intrinsic mannose patch is conserved across all gp120 proteins. However, we observed variations in both the size and composition of the intrinsic mannose patch. We show that there is a strong correlation between the frequency of outer-domain PNGSs and the abundance of oligomannose-type glycans for both CAP256 gp120s and a cross-clade panel of gp120s, highlighting the importance of the glycan density for the restricted access by glycan-processing enzymes. Although there were no strong correlations across the full time period in this donor, a general increase in total PNGSs was observed early in infection, and this increase correlated with an increase in oligomannose-type glycans. This was followed by a decline in PNGSs due to loss of glycans at the V3 base and a subsequent decline in oligomannose-type glycans, which was associated with the development of neutralizing antibodies to the C3V4 region. These results demonstrate the persistence of the intrinsic mannose patch over the course of HIV infection and further highlight this region as a stable target for HIV vaccine design strategies.

MATERIALS AND METHODS

Cloning and protein production. Cloning of the full-length soluble ectodomain of HIV-1 CAP256 gp120s (corresponding to amino acid residues 1 to 507, based on alignment to the HxB2 reference strain) into the pHLsec expression vector (50) has been described previously (16, 51). The CAP256 Env sequences were published previously (48, 49). The CAP256 proteins were expressed in the 293F variant of HEK 293T cells (ThermoFisher Scientific), which is adapted for suspension culture, in 500-ml Erlemeyer flasks with a vent cap (Corning). The cells were incubated at 37°C and 5% CO2, with shaking at 137 rpm as recommended by the manufacturer. Briefly, 200-ml cultures were transfected with plasmids (pHLSec) carrying the reporter gene expressing the protein using 293Fec-tin (ThermoFisher Scientific). The culture supernatants were harvested 5 days after transfection, and the His-tagged proteins were purified by Ni2+ affinity purification using a 5-ml HisTrap FF column (GE Healthcare). The nickel-purified proteins were further purified using size exclusion chromatography (SEC) on a Superdex 200 16/600 column (GE Healthcare). The monomeric fractions were collected, pooled, and analyzed using an SDS-PAGE 4% to 12% Bis-Tris NuPAGE gel (Invitrogen).

Glycan profiling by PNGase F release of N-glycans. N-glycans were released from target glycoprotein immobilized in SDS-PAGE bands using peptide-N-glycosidase F (PNGase F) (New England BioLabs) (52). Coo-massie-stained gel bands were excised and washed alternately with acetonitrile and water before being dried under vacuum. The gel pieces were rehydrated in 20 mM sodium bicarbonate buffer, pH 7.0, and incubated with PNGase F (1 μl) for 16 h at 37°C. The released glycans were extracted from the gel matrix by 3 washing steps with water.

Fluorescent labeling of N-linked glycans. The released glycans were subsequently fluorescently labeled and purified as previously described (33). The PNGase F-released N glycans were fluorescently labeled using 2-amino benzoic acid (2-AA). The labeling mixture comprised 2-AA (30 mg/ml) and sodium cyanoborohydride (45 mg/ml) dissolved in a solution of sodium acetate trihydrate (4% [wt/vol]) and boric acid (2% [wt/vol]) in methanol. The labeling mixture (80 μl) was added to each sample (in 30
µl of water) and incubated at 80°C for 1 h. The labeled oligosaccharides were purified using Spe-ed amide-2 columns (Applied Separations, Allen-town, PA) preequilibrated with acetonitrile. Before loading, 1 ml 97% (vol/vol) acetonitrile was added to each sample. The loaded samples were then washed with 2 ml 95% (vol/vol) acetonitrile and eluted with 1.5 ml water. The glycans were dried under vacuum prior to ultrasensitive liquid chromatography (UPLC) analysis or glycosidase treatment.

**Digestion of free labeled glycans.** Glycan samples labeled with 2-AA were digested overnight using endoglycosidase H (Endo H) (New England Bioscience) in a total volume of 20 µl. Samples were purified with a protein-binding membrane cleanup, using a Ludger vacuum manifold and a multiscreen filter protein-binding plate (Millipore).

**Hydrophilic interaction liquid chromatography-ultra-performance liquid chromatography.** Glycans were separated by hydrophilic interaction liquid chromatography (HILIC)-UPLC using a Waters Acuity system (Waters, USA). The labeled samples were resuspended in 15 µl water and added to a vial with 15 µl 100% acetonitrile. A 2:1-mm by 10-mm Acquity BEH amide column (Waters; particle size, 1.7 µm) with a programmed gradient was used for separation. Data were acquired and processed with Empower 3 (Waters, USA).

**Pseudovirus production and neutralization assays.** To produce pseudoviruses, plasmids encoding Env were cotransfected with an Env-deficient genomic backbone plasmid (pSG3ΔEnv) in a 1:2 ratio with the transfection reagent polyethyleneimine (PEI) (1 mg/ml; 1:3 PEI-total DNA; Polysciences) into HEK 293T cells (obtained from the American Type Culture Collection) (54, 55). Pseudoviruses were harvested 72 h posttransfection for use in neutralization assays. Neutralizing activity was assessed using a single-round replication pseudovirus assay with TZM-bl target cells (provided by John Kappes through the NIH AIDS Reagents Repository Program), as described previously (54, 55). Briefly, the anti-body was serially diluted in a 96-well flat-bottom plate and preincubated with virus for 1 h at 37°C. Cells at a concentration of 20,000/well were added to the virus-antibody mixture, and the luminescence was quantified 72 h following infection via lysis and addition of Bright-Glo Luciferase substrate (Promega). Dose-response curves were fitted using nonlinear regression (GraphPad Prism) to determine 50% inhibitory concentrations (IC_{50}).

**Antibodies.** PGT121, PGT128, PGT135, PG9, PGV04, VRC01, PGT151, and CAP256-VRC26.25 were transiently expressed with the pCDNA 3.1D-TOPO vector (Invitrogen) as described previously (56).

**Correlations and statistics.** Correlations were determined using a Pearson correlation and calculated using GraphPad Prism 6. For all CAP256 Envs, the frequency of gp41 PNGSs and the N289 or N295 glycan sites (Fig. 1B). In-terestingly, there was a general trend toward increasing numbers of PNGSs postinfection, a weak positive correlation (r = 0.21; P = 0.01) was observed. However, as the glycan shield is a dynamic entity that is under constant pressure from the host immune system, we also looked for correlations over shorter periods. In this donor, a strong positive correlation (r = 0.64; P < 0.0001) was observed until week 94, after which the number of PNGSs declined and the correlation weakened (Fig. 1B). This decrease corresponds predominantly to the loss of glycan sites at positions N295 and N332. A slight decrease in PNGSs around weeks 30 to 34 was also observed, which corresponded to loss of V1/V2 loop PNGSs and the N289 or N295 glycan sites (Fig. 1B). Interestingly, this is the first time point at which the V1/V2-specific antibody response was detected and subsequently led to a sudden increase in viral diversification (48, 57). A similar trend was observed for PNGSs on the OD (residues 252 to 482) of gp120 until week 94; however, there was no correlation over the full period (Fig. 1C). In summary, in the CAP256 donor, there was a general trend toward increasing numbers of PNGSs early in infection that decreased at the latest time point (week 176), which is consistent with previous studies (44, 45). However, there was still considerable variation between single viruses at a given time point (e.g., at week 176, the total numbers of PNGSs differed by 4 [Fig. 1B]), enabling us to assess the prevalence of the intrinsic mannose patch.

**Preparation of chimeric viruses.** Chimeric Env containing the C3V4 region were created using an overlapping PCR strategy and cloned into the pCDNA 3.1D-TOPO vector (Invitrogen) as described previously (56).

**RESULTS**

Longitudinal analysis of PNGSs and V loop lengths for CAP256 Env sequences. Env sequences from the CAP256 donor from multiple time points over the course of HIV infection have been reported previously (48, 49). Full Env single-genome amplification (SGA) and next-generation sequencing of the V1-to-V3 region (using the MiSeq platform) of viral variants from plasma samples correlated well (48, 57). Here, 154 clones from multiple time points were analyzed for their PNGS positions and frequencies, as well as their variable-loop lengths. Previous studies have reported an increase in PNGSs and variable-loop lengths over the course of HIV infec-

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monomeric gp120 was used as a useful model of this viral feature. Residues 1 to 517 were cloned into a recombinant expression vector (pHLSec) (50, 51) and expressed in HEK 293F cells for glycan profiling (we have previously shown that the mannose patch is largely independent of the producer cell [17, 18]). The protein constructs included a C-terminal hexahistidine tag so that nickel affinity purification could be used to avoid potential bias associated with other glycan-specific purification methods, such as lectins. Proteins were purified first using His tag affinity chromatography, followed by SEC to remove aggregates. The purified proteins were then run on a nonreducing SDS-PAGE gel, and the monomeric gp120 band was excised for glycan analysis. N-linked glycans were released using PNGase F, fluorescently labeled, and analyzed by HILIC-UPLC. The percentage of oligomannose glycans was assessed by integration of chromatograms pre- and post-Endo H digestion, generating specific percentage areas for the oligomannose glycans (Fig. 3A). It was then possible to assign structures based on previous analysis (16, 18).

All gp120 samples displayed an intrinsic mannose patch; however, the population of oligomannose-type glycans varied from 29.3% to 47.6% (see Table S1 in the supplemental material). The percent changes in oligomannose levels between gp120 94wks.A3, which has the highest number of PNGSs (a total of 28), and 6wks.PI, which has the fewest PNGSs (a total of 23), are 27% and 212% for Man5–9GlcNAc2 and Man9GlcNAc2, respectively. gp120 94wks.A3 has additional PNGSs in V1 (N135 and N160), in C2 (N230), in C3 (N362), and in V4 (N406, N413), whereas 6wks.PI has an additional PNGS in C4 (N442). We have previously measured the decrease in oligomannose-type glycans on BaL gp120 when one or two PNGSs were removed through Asn-to-Ala substitution (16). The largest effect was observed for the N295A/N386A double mutant, where the percentages of Man5–9GlcNAc2 and Man9GlcNAc2 decreased by 27% and 71%, respectively. Therefore, compared to our previous observations, the differences in oligomannose-type glycan abundances for 94wks.A3 and 6wks.PI are relatively small considering that these recombinant proteins differ by five PNGSs (16), but the difference in Man9GlcNAc2 structures is much higher. This observation was not unexpected, given that the positions of two of the additional PNGSs are on the gp120 OD, where PNGSs are tightly clustered (see Discussion below). This therefore suggests that there are regions on gp120 where multiple glycans can be removed with little impact on glycan processing of the intrinsic mannose patch and that it is the local density of PNGSs that determines the extent of glycan processing.

The abundance of oligomannose-type glycans correlates with the density of PNGSs. To determine factors that might influence the abundance of specific oligomannose-type glycans on gp120, we correlated the percentage of Man5–9GlcNAc2 glycans with the total number of PNGSs on gp120 (Fig. 3B). A positive correlation was observed ($r = 0.486; P = 0.016$), and this correlation became more significant when only Man9GlcNAc2 glycan abundance was considered ($r = 0.695; P = 0.0002$). When the percentages of Man5–9GlcNAc2 and Man9GlcNAc2 were corre-
lated with the frequency of PNGSs present only on the OD of gp120, a strong positive correlation was observed for both Man$_5$–9GlcNAc$_2$ and Man$_9$GlcNAc$_2$ ($r = 0.752$, $P < 0.0001$, and $r = 0.717$, $P < 0.0001$, respectively) (Fig. 3C). The gp120 OD PNGSs include many of the sites shown to be oligomannose type in site-specific analysis studies (8, 11, 14, 22, 60). Further, the recent crystal structures of the BG505 SOSIP.664 recombinant trimer showed that the PNGSs in the region cluster tightly on the surface of Env (58, 61). Therefore, an increase in PNGSs on the outer domain of gp120 will likely further restrict access of the glycan-processing enzymes, leading to an increase in oligomannose-type glycans on gp120 and in the size of the intrinsic man-
Longitudinal Persistence of the HIV Mannose Patch

FIG 3 (A) HILIC-UPLC spectrum of fluorescently labeled N-linked glycans released from 48wks.17 gp120 using PNGase F presented as an example of the quantification methodology. The green trace is a spectrum of released glycans, and the white trace is the spectrum for Endo H-treated glycans. Overlaying of the spectra resulted in the glycans sensitive to Endo H being displayed as green. M5–M9 refers to Man₅–₉GlcNAc₂. (B to D) The percentage of oligomannose glycans was assessed by integration of chromatograms pre- and post-Endo H digestion, generating specific percentage areas for the oligomannose glycans. The oligomannose glycans are highlighted. Shown are correlations between the abundances of oligomannose-type glycans (Man₅₋₉GlcNAc₂ and Man₉GlcNAc₂) and total PNGSs on gp120 (B), PNGSs on the gp120 outer domain (residues 252 to 482), and total variable-loop lengths (D). The correlations were assessed by Pearson analyses; P values and r values are indicated.

The abundance of oligomannose-type glycans correlates with the density of outer-domain PNGSs for a cross-clade panel of gp120s. To determine whether the correlation between the number of PNGSs on the outer domain of gp120 and the abundance of oligomannose-type glycans was a general feature for HIV Env across geographical clades, a panel of 29 gp120s were cloned, expressed, and purified as described above. The panel included gp120s from clades A, B, C, AE, and G, five of which were transmitted/founder viruses. All the isolates tested were found to possess a significant population of oligomannose-type glycans ranging from 23.8% to 50.5% (Fig. 4; see Table S2 in the supplemental material). When the abundance of oligomannose-type glycans was correlated with the total number of PNGSs (ranging from 21 to 28), no significant correlation was observed (Fig. 4A). However, a significant correlation was observed between the frequency of OD PNGSs (ranging from 12 to 17) and oligomannose abundance, similar to that seen for the CAP256 samples (r = 0.4692; P = 0.010) (Fig. 4B). No significant correlations were observed between total PNGSs or OD PNGSs and Man₉GlcNAc₂ (data not shown). These data further support the notion that a high density of PNGSs on the OD restricts glycan-processing enzymes, leading to a larger population of underprocessed oligomannose-type glycans. These data also suggest that it is local glycan density that has the largest impact on glycan processing, rather than overall glycan density. Interestingly, the specific occupancy and composition of individual sites was not assessed here, but this could be an informative extension in future studies.

While there are variations in the percentages of certain oligomannose structures between the clades (clades C and G have fewer Man₉GlcNAc₂ structures, and clade C has fewer Man₆GlcNAc₂ structures), the overall abundances of oligomannose glycans are fairly similar (Fig. 4C and D). Clade C has the lowest total percentage of oligomannose (35.5%), yet compared to clade B, with one of the highest percentages and lowest standard deviation (SD) (38.5%; SD, 4.98), there is no significant difference between the two (Fig. 4C), although this difference might become more significant if more gp120s were studied. Considering the correlation between outer-domain PNGSs and Man₅₋₉GlcNAc₂, it is likely that loss of specific sites between clades is responsible for the differences in specific glycan abundance. This is particularly relevant for clade C viruses, which typically lack the N295 glycan site (62, 63), a PNGS we have previously shown to stabilize the mannose patch from glycan processing (16). However, while there are some differences in the structures, the total levels of oligomannose-type glycans remain similar between clades, indicating the overall stability and conserved nature of the mannose patch.

Correlation of oligomannose-type glycans with time postinfection. We next examined how the size of the intrinsic mannose patch changes over the course of HIV infection. We first corre-
lated the percentage of oligomannose-type glycans with the number of weeks postinfection, but no correlation was observed (Fig. 5A). As the glycan shield is a dynamic entity that is under constant pressure from the host immune system, we also looked for correlations over shorter periods to reflect this. We observed correlations between Man5–9GlcNAc2 and Man9GlcNAc2 abundance and the number of weeks postinfection until week 94 ($r = 0.513$, $P = 0.025$, and $r = 0.666$, $P = 0.0019$, respectively) (Fig. 5A) similar to that seen for changes in PNGS frequency over time. The abundance of oligomannose-type glycans, then, largely persists but exhibits some variation due to sensitivity to loss of PNGSs at the base of V3, in particular at positions N295 and N332/N334 (Fig. 2).

To analyze the changes in Env glycan composition over time in more detail, we next determined the percent change in total oligomannose-type glycans (Man5–9GlcNAc2) and Man9GlcNAc2 individually for each gp120 clone (Fig. 5B). As the viral population early in infection was predominantly made up of the SU virus, with only the V1/V2 and gp41 C terminus mostly being derived from the PI virus, changes in total Man$_{5-9}$GlcNAc$_2$ and Man$_9$GlcNAc$_2$ composition were considered in relation to the SU virus. Although the mannose patch is present on all CAP256 proteins studied, the changes in Man$_{5-9}$GlcNAc$_2$ and Man$_9$GlcNAc$_2$ composition can vary for individual clones at a given time point. Generally, a large increase in oligomannose-type glycans was due to increases in Man$_9$GlcNAc$_2$ and Man$_9$GlcNAc$_2$ early-stage glycan structures (Fig. 5B; see Table S1 in the supplemental material), further suggesting that increased density in PNGSs leads to reduced glycan processing. For example, gp120s from weeks 59 and 94, which had the highest oligomannose-type glycan abundance (44.0 to 47.6%), had 28.4% to 30.4% Man$_{8-9}$GlcNAc$_2$ structures. Interestingly, previous analysis of glycan site mutants showed that the presence of Man$_9$GlcNAc$_2$ was particularly dependent on multiple stabilizing interactions with neighboring glycans (16).

The abundance of oligomannose-type glycans does not correlate with the neutralization potency of anti-HIV bnAbs. We next wanted to determine whether the structure of the HIV glycan shield, in particular the abundance of oligomannose-type glycans, might influence the potency of neutralization by a panel of HIV bnAbs. We therefore determined the IC$_{50}$s for the intrinsic mannone patch binding bnAbs PGT121, PGT128, and PGT135; the V1/V2 loop bnAb PG9 and several members of the CAP256-VRC26 antibody lineage; cleavage-specific bnAb PGT151; and CD4 binding site bnAbs PGV04, VRC01, and llama antibody VHH J3 (see Table S3 in the supplemental material). When the IC$_{50}$s were correlated with the abundances of oligomannose-type glycans, no significant correlations were observed for any bnAbs (Fig. 6), although a general weak trend for increasing IC$_{50}$s with increasing oligomannose-type glycans was observed for some bnAbs. Generally, the ability of a bnAb to neutralize a viral variant was dependent on the presence of key contact glycan sites, such as N160 or N332. The majority of viruses were resistant to PGT135 neutralization, and viruses lacking the N332 glycan site, and in
one case the N295 glycan site, were resistant to PGT128. PGT121 was able to neutralize all but two viruses. PG9 could not neutralize viruses lacking the N160 glycan site or viruses with a glutamic acid at position 169 (Fig. 2 and 6; see Table S3 in the supplemental material) (48), whereas CAP256-VRC26 lineage bnAbs were dependent on protein residues in V1 for neutralization (27). Interestingly, none of CAP256-VRC26 lineage bnAbs isolated over several different time points throughout infection (weeks 119, 159, 193, and 206) showed any correlation with oligomannose abundance, suggesting that increasing the size of the mannose patch is not a direct mechanism of escape against the autologous antibodies in this donor. For PGT151, although all the viruses contained the key glycan sites and residues thought to be required for neutralization (N611, N637, and E647), some viruses were nonetheless resistant to PGT151 neutralization. The potencies for the CD4 binding site bnAbs PGV04, VRC01, and J3, which do not contact glycans, generally did not correlate with the levels of oligomannose-type glycans. As N-linked glycans are positioned around the edge of the CD4 binding site, the changes in bulk glycan structures observed may not occur in this region of gp120 and therefore may not impact CD4 binding site bnAbs, but site-specific glycan analysis would be required to determine this. Interestingly, the smaller single-chain llama antibody, J3, had the smallest variation in IC50s. Therefore, the abundance of oligomannose-type glycans in the intrinsic mannose patch does not impact the potency of neutralization and suggests Env sequences from any time point during infection, provided they have the key contact glycan sites, would be suitable HIV immunogens.

Anti-C3V4 nAbs may be responsible for loss of PNGSs at week 176. The loss of glycan sites at the base of the V3 loop at week 176 suggested that neutralizing antibodies might exert selection pressure against this region that leads to loss of
sequences from a chronically infected HIV patient to characterize the changes in the structure of the HIV glycan shield during the course of HIV infection, in particular the persistence and composition of the intrinsic mannose patch. We showed that in the CAP256 donor, the mannose patch (Fig. 8A) persists throughout infection despite the variation in PNGS position and frequency (Fig. 8B). In this donor, there is an increase in PNGSs and oligomannose-type glycans within the intrinsic mannose patch over the course of infection until week 94. This increase correlates with the frequency of PNGSs on the outer domain. Thereafter, there is a reduction in PNGSs at the base of V3 and a corresponding reduction in oligomannose-type glycans by week 176, likely a consequence of viral escape from a de novo neutralizing response to the C3V4 region. Although this study focuses on only one donor, these findings give insight into the composition and conservation of the intrinsic mannose patch under immune pressure and highlight the epitope as an important target for HIV vaccine design strategies.

Our previous studies have shown that the glycosylation of HIV Env is determined by both protein-directed effects, arising from the 3-dimensional protein structure, and cell-directed effects, arising from the cell type the protein is expressed in (2, 17, 18). The protein-directed effects give rise to a patch of underprocessed oligomannose-type glycans on the outer domain of gp120 that forms a nonself epitope targeted by HIV bnAbs. We show that despite the variation in the protein sequence and the positioning and frequency of PNGSs, the intrinsic mannose patch is highly conserved during the course of infection in the CAP256 donor and therefore represents a stable target for vaccine design. However, the intrinsic mannose patch varies in both overall size and distribution of glycans within the oligomannose series (Man$_{3-5}$GlcNAc$_2$), and this most strongly correlates with the density of PNGSs present on the OD of gp120. This trend was also observed, although to a lesser extent, for a cross-clade panel of gp120s and highlights the role the protein sequence might also play in determining the structure of the HIV glycan shield. These data support our previous conclusions that the high density of PNGSs restricts glycan-processing enzymes from trimming and processing N-linked glycans within the region (7, 15, 16, 18, 64). Interestingly, it seems to be the local glycan density rather than the overall glycan density that has the biggest impact on the size and composition of the mannose patch. Although Env se-

PNGSs and a decrease in abundance of oligomannose-type glycans. To assess whether the region was a target of nAbs, we created a chimeric Env from the 176wks.4 Env, which had already escaped the high-titer V2 responses that dominate CAP256 plasma (49). Using overlapping PCR, we transferred the C3V4 region from the sensitive 15wks SU virus into the resistant backbone and tested this chimeric Env (15wks SU C3V4) against longitudinal plasma (Fig. 7). Anti-C3V4 antibodies at titers greater than 1:100 were detected from 42 weeks postinfection and persisted at least until 94 weeks postinfection. The anti-C3V4 antibodies show some N332A dependence. Titers are indicated as plasma 50% infective dose (ID$_{50}$) versus number of weeks postinfection.

**DISCUSSION**

It is clear that the HIV glycan shield is under constant pressure from the host immune system. Here, we used longitudinal Env sequences from a chronically infected HIV patient to characterize the changes in the structure of the HIV glycan shield during the course of HIV infection, in particular the persistence and composition of the intrinsic mannose patch. We showed that in the CAP256 donor, the mannose patch (Fig. 8A) persists throughout infection despite the variation in PNGS position and frequency (Fig. 8B). In this donor, there is an increase in PNGSs and oligomannose-type glycans within the intrinsic mannose patch over the course of infection until week 94. This increase correlates with the frequency of PNGSs on the outer domain. Thereafter, there is a reduction in PNGSs at the base of V3 and a corresponding reduction in oligomannose-type glycans by week 176, likely a consequence of viral escape from a de novo neutralizing response to the C3V4 region. Although this study focuses on only one donor, these findings give insight into the composition and conservation of the intrinsic mannose patch under immune pressure and highlight the epitope as an important target for HIV vaccine design strategies.

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quences vary by up to 5 PNGSs, it is clear that it is mainly PNGSs within and around the outer domain of gp120 that affect the size and distribution of oligomannose-type glycans within the intrinsic mannose patch. The potency of neutralization by a panel of HIV bnAbs is not affected by the variation in mannose patch composition but is dependent on the presence of certain key PNGSs. This suggests that PNGSs on gp120 have sufficiently high density that the natural variation in Env occurring throughout infection has minimal impact on glycan processing, so that the mannose patch, which is intrinsic to both the monomer and the trimer, is always present. Therefore, the density of glycans on gp120, even at the lowest density, is sufficient to maintain the steric restriction necessary to impede mannosidase processing. This is consistent with previous observations suggesting that minimal glycan-glycan interactions are required to prevent processing to complex-type glycans (16). In addition, this effect may be further compounded by the trimer-associated restriction of processing not captured by our monomeric gp120 model (1, 2, 18, 22). Although several studies have reported more compact transmitter clade C and A viruses (39, 40), with shorter V1-to-V4 loop lengths, this does not appear to impact the glycosylation of gp120s from the CAP256 donor.

The most dramatic changes to the HIV glycan shield of CAP256 gp120 occur when glycans at the base of the V3 loop are added or deleted. This is supported by our previous studies showing that deletion of glycans within this region for gp120$_{cd4}$ had the largest impact on oligomannose-type glycan abundance due to disruption of glycan microclusters within the outer domain (16). We have shown that some of the changes occurring in the PNGS position and frequency of CAP256 gp120, and subsequently oligomannose abundance, at week 176 postinfection are likely a result of a new wave of neutralizing antibodies targeting the C3V4 region, including the N332 glycan. These data may suggest that the selective pressure of neutralizing Abs targeting the intrinsic mannose patch would have the biggest effect on shaping the glycan structures present on the HIV glycan shield. Unfortunately, full-length Envs from later time points were not available, but as the C3V4-specific response arose after approximately 75 weeks, any additional destabilization of the intrinsic mannose patch is likely to occur within the time frame studied. It is possible that if a similar study were carried out in a donor who developed bnAbs against another epitope, such as the CD4 binding site, less variation in OD PNGS frequency would occur, and thus, a smaller variation in oligomannose-type glycans would be observed over the course of infection.

Go and colleagues have previously compared the glycosylation of recombinant gp120 from transmitted/founder (t/f) viruses and chronic viruses (60). They concluded that t/f virus Envs are more similar to each other than to those of their corresponding chronic viruses, with t/f Envs having distinct glycosylation patterns consisting of higher levels of oligomannose and sialylated glycans and a lower site occupancy (60). However, the study was limited, as only two t/f and two chronic viruses were studied, and these viruses were not derived from the same donors. Indeed, comparison of oligomannose levels on the t/f and chronic viruses in our gp120 panel showed no significant differences. By using longitudinal virus sequences, we were able to show that over the course of infection in the CAP256 individual, there was an increase in PNGSs and a corresponding increase in oligomannose-type glycans that were subsequently reduced by the pressure of neutralizing antibodies. Although the PI and SU virus gp120s have lower levels of oligomannose-type glycans (35.3% and 36.4%, respectively) than the majority of gp120s from later time points, there are viruses within the quasispecies that have lower levels of oligomannose glycans, e.g., 38wks.38 and 48wks.10, with 32.7% and 35.5% oligomannose-type glycans, respectively. It would be interesting to determine the glycosylation of Envs within the HIV-infected donor who transmitted the viruses to the CAP256 donor; however, these samples are not available.

Although we have studied only one HIV-infected individual in detail, a number of studies have shown that t/f viruses have a lower frequency of PNGSs (39–43). Whether there would be a benefit for t/f viruses to have a reduced frequency of PNGSs and subsequently to display a lower proportion of oligomannose-type glycans is unclear. In relation to HIV transmission, studies have shown that the importance of the interaction of DC-SIGN receptors on dendritic cells (DCs) in mucosal tissues for transfection of CD4$^+$ T cells is strongly dependent on the presence of oligomannose structures (65–67). In relation to infectivity, reduction of complex-type glycans on HIV virions (through the use of glycosidase inhibitors or a GnTI-deficient cell line) reduced the infectivity of the virus but enhanced transfection of peripheral blood lymphocytes (32, 68). In relation to Env immunogenicity, studies have shown that removal or occlusion of mannose residues from the surface of gp120 can enhance the immune response against HIV due to reduced interactions with immunosuppressive receptors, such as the mannose receptor (69–71). Taken together, these studies might suggest that a higher abundance of oligomannose-type glycans would be more beneficial for transmitted viruses. It is therefore possible that the reduced oligomannose levels in the PI and SU viruses are only a consequence of fewer PNGSs and do not give a virus a competitive advantage at the point of transmission. However, there may be a tradeoff between viral infectivity and host recognition. Regardless, in terms of vaccine design, Env-based immunogens with a lower abundance of oligomannose-type glycans (for the CAP256 donor, this would be Envs from earlier time points) might give a stronger immune response, as suggested by the studies described above (69–71).

In summary, although in the CAP256 donor there were changes in both the frequency and positioning of PNGSs due to immune pressure, the intrinsic mannose patch remained a stable feature of HIV Env and was present throughout the course of HIV infection. The density of PNGSs on the outer domain of gp120 can influence the size and composition of the intrinsic mannose patch, but these differences do not affect the neutralization sensitivity of a panel of HIV bnAbs. These findings, in addition to our previous observations showing the presence of the intrinsic mannose patch to be independent of producer cells, further highlight the mannose patch as a stable target for HIV vaccine design.

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